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PMID: 8128456 [PubMed - indexed for MEDLINE]

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Characterization of phospholipase A2 secretion from human platelets.

Mueller HW, Pritzker CR, Kubik A, Deykin D.

Boston VA Medical Center, Massachusetts.

Human platelets secreted phospholipase A2 in a dose- and time-dependent manner when challenged with thrombin, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), or collagen. Enzyme release was maximal at concentrations of 0.1 units/ml of thrombin, 100 nM TPA, or 2 micrograms/ml of collagen; and complete by 2 min in platelets treated with thrombin or TPA. Cells challenged with collagen required up to 5 min for maximal secretion. Besides dose and time functions, phospholipase A2 secretion was also dependent on platelet concentration and the levels of bovine serum albumin in the incubation medium. The secreted enzyme was soluble and exhibited substrate and Ca2+ requirements similar to a detergent-solubilized, partially purified phospholipase A2 from whole platelets [Kramer et al., Biochim. Biophys. Acta (1988) 959, 269-279]. The pH optimum of the secreted enzyme, however, was 1-2 units lower than the pH optimum of the phospholipase A2 from whole cells. Secreted phospholipase A2 hydrolyzed phosphatidylethanolamine at 5-12 times the rate of phosphatidylcholine when the substrates were present in pure form. These apparent differences in activity were greatly diminished, though, when 1:1 molar mixtures of the two substrates were employed. Because phospholipase A2 catalyzes a key reaction during the formation of bioactive arachidonate metabolites, the secretion of this enzyme from platelets may be important in the regulation of thrombosis.

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CHARACTERIZATION OF PHOSPHOLIPASE A_2 SECRETION FROM HUMAN PLATELETS

Howard W. Mueller, Carole R. Pritzker, Anne Kubik and Daniel Deykin
Boston VA Medical Center, Boston, Massachusetts, and Division of Cardiology, University of Texas Health Science Center, Houston, Texas

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Abstract Human platelets secreted phospholipase A_2 in a dose- and time-dependent manner when challenged with thrombin, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), or collagen. Enzyme release was maximal at concentrations of 0.1 units/ml of thrombin, 100 nM TPA, or 2 μg/ml of collagen; and complete by 2 min in platelets treated with thrombin or TPA. Cells challenged with collagen required up to 5 min for maximal secretion. Besides dose and time functions, phospholipase A_2 secretion was also dependent on platelet concentration and the levels of bovine serum albumin in the incubation medium. The secreted enzyme was soluble and exhibited substrate and Ca^{2+} requirements similar to a detergent-solubilized, partially purified phospholipase A_2 from whole platelets [Kramer et al., Biochim. Biophys. Acta (1988) 959, 269-279]. The pH optimum of the secreted enzyme, however, was 1-2 units lower than the pH optimum of the phospholipase A_2 from whole cells. Secreted phospholipase A_2 hydrolyzed phosphatidylethanolamine at 5-12 times the rate of phosphatidylcholine when the substrates were present in pure form. These apparent differences in activity were greatly diminished, though, when 1:1 molar mixtures of the two substrates were employed. Because phospholipase A_2 catalyzes a key reaction during the formation of bioactive arachidonate metabolites, the secretion of this enzyme from platelets may be important in the regulation of thrombosis.

A primary function of blood platelets is to maintain the integrity of the systemic circulation by forming cellular aggregates at sites of vascular injury. One biochemical

Key words: Phospholipase A_2, platelet, human, secretion, arachidonic acid.
Abbreviations: GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGI_2, prostacyclin; PLA_2, phospholipase A_2; TLC, thin-layer chromatography; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; TXA_2, thromboxane A_2.
Corresponding author: Dr. Howard W. Mueller, Division of Cardiology, Univ. of Texas Health Science Center, 6431 Fannin St., Houston, TX 77030

mechanism by which platelet activation occurs is through the endogenous production of thromboxane A_2, a potent inducer of platelet aggregation and smooth muscle contraction. The pro-thrombotic effects of TXA_2 are directly opposed by prostacylin, an eicosanoid product of vascular endothelial cells which inhibits platelet aggregation and promotes smooth muscle relaxation (1-4). Both TXA_2 and PGI_2 are synthesized from arachidonic acid by prostaglandin endoperoxide synthase, a protein which contains both a cyclooxygenase and a peroxidase activity. These two activities convert arachidonate to the cyclic endoperoxides, prostaglandin G_2 and prostaglandin H_2, respectively. Prostaglandin H_2 is subsequently converted to TXA_2 by thromboxane synthase in the platelet, or to PGI_2 by PGI_2 synthase in the endothelial cell (5).

The arachidonic acid utilized for TXA_2 production is not free in the cell, but rather is esterified at the *sn*-2 position of membrane glycerophospholipids. Upon cellular activation, this arachidonate is released in a key reaction catalyzed by phospholipase A_2 (2,6,7). Although classically, platelet PLA_2 is thought to act intracellularly to hydrolyze endogenous phospholipid substrates, recent studies with sheep (8), rat (9), and rabbit (10) platelets have demonstrated that these cells also secrete this enzyme upon stimulation. Since secreted PLA_2 could act extracellularly to liberate arachidonate in other platelets or vascular endothelial cells, the release of this enzyme offers a potentially novel dimension for the regulation of hemostatic and thrombotic events.

In the present study, we examined the secretion of PLA_2 from human platelets. Although PLA_2 secretion from human platelets was not observed in a previous study (10), this work characterizes the release of PLA_2 from these cells in response to several physiological and non-physiological stimuli. Data regarding the substrate, Ca^{2+}, and pH requirements of the secreted enzyme are also presented.

EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin (essentially fatty acid-free) and 12-*O*-tetradecanoyl-phorbol-13-acetate were purchased from Sigma; the TPA was dissolved in dimethyl sulfoxide prior to use. 1-Stearoyl-2-arachidonyl-GPC, 1-stearoyl-2-arachidonyl-GPE, and phospholipid standards were purchased from Avanti Polar Lipids. Arachidonic acid was obtained from Nu-Chek Prep and Silica Gel G TLC plates were purchased from Analtech. Equine collagen was obtained from Horm-Chemie and human α-thrombin was a gift from Dr. John W. Fenton. 1-Stearoyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine (91 Ci/mmol) was purchased from New England Nuclear; this compound was used to synthesize 1-stearoyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphoethanolamine in the presence of partially purified cabbage phospholipase D and free ethanolamine (11). Briefly, cabbage phospholipase D was purified through Stage 3 by the method of Davidson and Long (12). A small aliquot of the enzyme preparation was combined 1:1 (v/v) with a solution containing 160 mM sodium acetate (pH 5.6), 80 mM calcium chloride, and 15% ethanolamine. One ml of enzyme-buffer mixture was then added to 1 ml of diethyl ether containing the substrate, 1-stearoyl-2-[^{3}H]arachidonyl-GPC (20-40 μCi), and the mixture was shaken vigorously at room temperature for 1 hour. The reaction was stopped by total lipid extraction (13), and product formation was confirmed by zonal TLC analysis of the chloroform extract on Silica Gel G using a solvent system of chloroform:methanol:NH_4OH (65:35:8, v/v). The

[^{3}H]PE was finally purified by normal phase HPLC (14) and stored at -20°C in chloroform:methanol (4:1, v/v). The product yield from this reaction was 40-50%.

Isolation of Human Platelets

Blood was drawn from healthy donors into a 1/6 volume of acid citrate dextrose (85 mM trisodium citrate, 64 mM citric acid, and 111 mM glucose) and platelet-rich plasma was prepared by centrifugation at 170 x g for 15 min. The platelet-rich plasma was removed and the cells were sedimented by centrifugation at 1,870 x g for 15 min. The platelet pellet was washed twice in a gel filtration buffer (15) (pH 6.5) containing 129 mM NaCl, 10.9 mM sodium citrate, 8.9 mM sodium bicarbonate, 1 mg/ml glucose, 10 mM Trizma base, 2.8 mM KCl, 0.8 mM KH_2PO_4, 2 mM EDTA, and 1 mg/ml BSA; and the cells were resedimented by centrifugation at 830 x g for 15 min. The washed platelet pellet was finally resuspended in gel filtration buffer (pH 7.5) without EDTA, or in later experiments in a HEPES-buffered medium (9) (pH 7.5) containing 137 mM NaCl, 2.7 mM KCl, 10 mM HEPES, and 0.1% glucose. The use of the HEPES buffer during cell challenge resulted in a 35-40% increase in secreted phospholipase activity. In some experiments, platelets were also obtained from two-day-old platelet concentrates from the Red Cross. These cells were isolated and washed in the same manner as described for fresh platelet-rich plasma above. All platelet preparation steps were performed at room temperature.

Incubation Procedures

Platelets at a final concentration of 0.5-1 x 10^9 cells/ml were incubated in a shaking water bath at 37°C for 0-15 min in gel filtration buffer or HEPES-buffered medium (pH 7.5) containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml BSA, and the indicated stimuli. In later experiments using thrombin as a stimulus, the Ca^{2+} and Mg^{2+} were omitted and replaced with 1 mM EDTA without any loss of secreted PLA_2 activity. The reactions were stopped by placing the cells in an ice water bath for 10 min, and the cells were sedimented by centrifugation at 830 x g and 4°C for 10 min. The supernatants were removed and centrifuged at 2,400 x g and 4°C for 15 min to remove any unsedimented cells, and then assayed for phospholipase activity.

Assay of Secreted Phospholipase A_2

Secreted PLA_2 activity was assayed as described by Kramer et al. (16) with slight modifications. Routinely, 1-stearoyl-2-[^{3}H]arachidonyl-GPC or -GPE (100,000 dpm/tube; 0.5 pmol/tube) was dried under N_2 and sonicated on ice in 100 mM Tris buffer (pH 7.5-9.0); $CaCl_2$ and BSA were then added to concentrations of 10 mM and 1 mg/ml, respectively. The recovery of radiolabeled phospholipid in the buffer was increased from 60% to approximately 95% when the calcium was added after sonication. The sonicated substrate and platelet supernatant were combined 1:1 (v/v, 150 μl of each) for final $CaCl_2$ and BSA concentrations of 5 mM and 1 mg/ml, respectively, and the mixture was incubated for 1 hour at 37°C. The reactions were stopped by addition of Dole's reagent (17), and the radiolabeled fatty acid product was selectively extracted into heptane (16) and measured by liquid scintillation counting. In some experiments, the reactions were stopped by addition of chloroform:methanol (2:1, v/v) and the total lipids were extracted (13). The levels of labeled fatty acid product were then measured following separation by TLC on Silica Gel G plates developed in hexane:diethyl ether:glacial acetic acid (60:40:1, v/v). In experiments designed to measure the pH optima of the secreted PLA_2, a buffer containing

100 mM glycylglycine, 100 mM piperazine, and 100 mM sodium formate (pH 2-11) was substituted for the Tris buffer.

Other Methods

Ionized calcium measurements were performed using a Calcium Selectrode from Radiometer. A calcium titration curve of electrode response (mV) vs. added calcium (μmol) was established by titrating known amounts of $CaCl_2$ into a buffer solution which mimicked the assay conditions; this solution contained 25 ml of HEPES buffered medium (pH 7.5) with 1 mM EDTA and 25 ml of 100 mM Tris buffer (pH 7.5). An equation for the curve, which was generated using Prophet curve-fitting software, was then used to calculate the amount of $CaCl_2$ required for a given ionized calcium level. Lipid phosphorus measurements of the unlabeled phospholipid substrates were performed by the method of Rouser et al. (18).

RESULTS

Agonist-Stimulated Secretion of Phospholipase A_2 from Human Platelets

When platelets were challenged with thrombin, TPA, or collagen and the extracellular medium was subsequently assayed for phospholipase A_2, a dose-dependent release of activity was observed (Fig. 1). The secretion of PLA_2 was maximal at concentrations of 0.1 units/ml of thrombin, 100 nM TPA, or 2 μg/ml of collagen. When the relative potencies of these stimuli were examined on the same preparation of platelets, TPA was the most effective agonist for eliciting PLA_2 release, followed by thrombin (80% of TPA) and collagen (66% of TPA) (data not shown). Time course experiments also shown in Fig. 1 demonstrated that PLA_2 release was complete by 2 min in cells challenged with thrombin or TPA; however, collagen-treated platelets required up to 5 min before maximal release was attained.

The secretion of phospholipase A_2 by human platelets was also dependent on cell concentration. As illustrated in Fig. 2, enzyme release increased as a function of platelet concentration up to 5×10^8 cells/ml, afterwhich a plateau in secreted activity was observed. Interestingly, control platelets also secreted PLA_2 when incubated at high cell densities, presumably due to the accumulation of low levels of an endogenous agonist such as ADP or thromboxane A_2. Besides cell concentration, PLA_2 release was also affected by the concentration of BSA in the challenge medium. As shown in Fig. 3, the presence of BSA at concentrations $\geq$ 0.5 mg/ml resulted in a 2.5-fold enhancement of secreted activity. It should be noted that during this experiment, the BSA concentrations of all points were adjusted to the same level (2.83 mg/ml) prior to PLA_2 assay. Therefore, Fig. 3 reflects only the effect of BSA on enzyme secretion and not enzyme activity.

Characterization of Secreted Phospholipase A_2

To determine if the secreted PLA_2 was soluble or associated with a particulate fraction, supernatants derived from control, thrombin-, TPA-, or collagen-treated platelets were centrifuged at 100,000 x g for 1 hour. Parallel samples were centrifuged under routine conditions at 2,400 x g for 15 min. When the PLA_2 activities in the high- and low-speed supernatants were assayed and compared, no loss of activity in the high-speed supernatants was observed, demonstrating that the enzyme is soluble (data not shown).

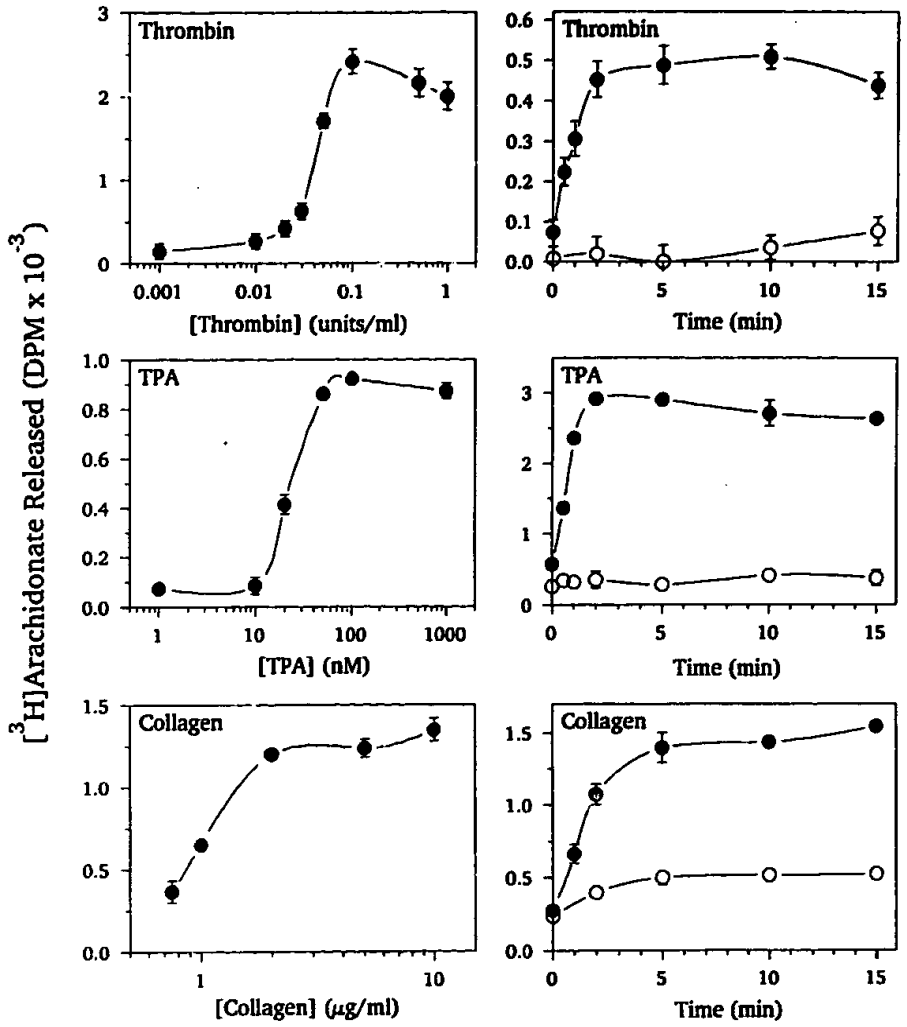
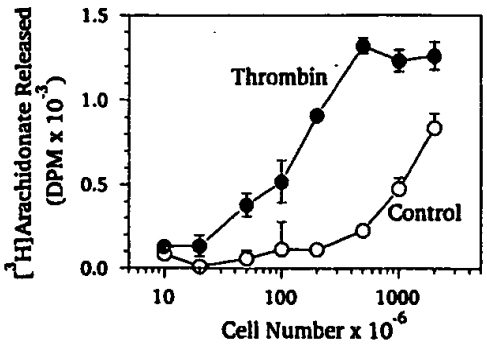


FIG. 1

Dose- and time-dependent secretion of phospholipase A_2 by human platelets stimulated with thrombin, TPA, or collagen. Left-Hand Panels: As detailed under "Experimental Procedures", fresh platelets (3.6-7.1 x 10^8 cells/ml) were incubated for 5 min at 37°C in gel filtration buffer containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml BSA, and the indicated concentrations of stimuli. Right-Hand Panels: Platelets were incubated for the indicated times in the same buffer containing one of the following additions: buffer (control), 0.1 units/ml of thrombin, 100 nM TPA, or 5 μg/ml of collagen. The supernatants from both sets of experiments were then assayed in duplicate for phospholipase A_2 activity using 1-stearoyl-2-[^{3}H]arachidonyl-GPC as substrate. The data, which are presented as the assay mean ± standard deviation, are representative of 4 separate dose experiments using thrombin, 2 dose experiments using TPA, and 3 dose experiments using collagen. The time course data are representative of 2 experiments using thrombin, 2 experiments using TPA, and 1 experiment using collagen as a stimulus. Closed circles, with stimulus; open circles, without stimulus.

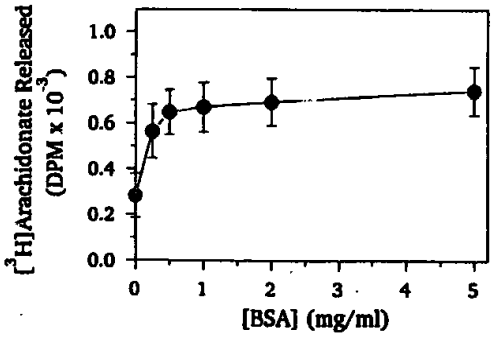
FIG. 2



Effect of platelet concentration on phospholipase A_2 secretion. Fresh platelets were incubated for 10 min at the indicated cell concentrations in gel filtration buffer containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml BSA, and either 0.5 units/ml thrombin (closed circles) or buffer alone (control, open circles). The supernatants were then assayed in duplicate for phospholipase A_2 activity using 1 stearoyl-2-[^{3}H]arachidonyl-GPC as substrate. These data, which are presented as the assay mean ± standard deviation, are representative of 3 separate experiments.

In experiments to characterize further the secreted phospholipase A_2, the effects of substrate concentration, Ca^{2+} concentration, and pH on the enzyme were evaluated. When 1-stearoyl-2-[^{3}H]arachidonyl-GPC was employed as substrate, the enzyme's activity approached saturation at phospholipid concentrations above 2 μM. Similar substrate concentration dependency was also observed in preliminary experiments utilizing 1-stearoyl-2-[^{3}H]arachidonyl-GPE (data not shown). The secreted PLA_2 exhibited an absolute requirement for calcium and a biphasic response during Ca^{2+} titration experiments (Fig. 4). At Ca^{2+} levels of 20-100 μM, a plateau of enzyme activity was observed representing 60% of maximal hydrolysis. As the calcium concentration was increased further, a second plateau of PLA_2 activity was seen at Ca^{2+} levels ≥ 1 mM. This biphasic reponse to calcium concentration has also been observed with phospholipase A_2 partially purified from detergent-solubilized whole platelets (19). The effect of pH on secreted PLA_2 is shown in Fig. 5. The enzyme, which had a pH optimum of 7.5-8, exhibited significant activity over a pH range of 6.5-10.

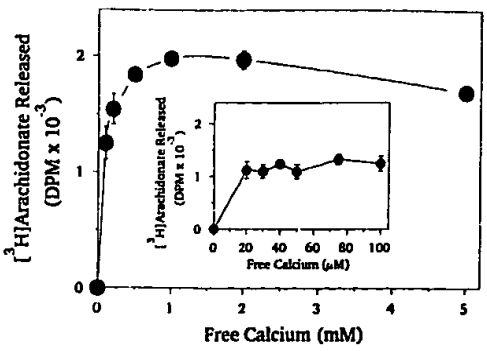
FIG. 3



Enhancement of phospholipase A_2 secretion by BSA. Fresh platelets (4.9 x 10^8 cells/ml) were incubated for 5 min in gel filtration buffer containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 units/ml thrombin, and the indicated concentrations of BSA. The supernatants were then assayed in triplicate for phospholipase A_2 activity using 1-stearoyl-2-[^{3}H]arachidonyl-GPC as substrate; the concentration of BSA during the assay was 2.83 mg/ml for all points. The data are presented as the assay mean ± standard deviation.

FIG. 4

Effect of Ca^{2+} concentration on secreted PLA_2 activity. Red Cross platelets (9.2 x 10^8 cells/ml) were incubated for 5 min in HEPES-buffered medium containing 0.5 units/ml thrombin, 1 mM EDTA, and 1 mg/ml BSA. The supernatant from this reaction was then assayed in triplicate for phospholipase A_2 activity in the presence of the indicated concentrations of Ca^{2+}. The data, which are presented as the assay mean ± standard deviation, are representative of 4 experiments.



To determine the effect of the substrate's polar head group on secreted PLA_2 activity, the relative hydrolysis of 1-stearoyl-2-[^{3}H]arachidonyl-GPC and 1-stearoyl-2-[^{3}H]arachidonyl-GPE were compared. As shown in Table I, PE hydrolysis was 5-12 times greater than PC hydrolysis when the substrates were present in pure form. However, since pure PC and PE vesicles have different physical structures (bilayer vs. hexagonal H_{II} phase) in an aqueous environment (20), the enhanced activity observed with PE could be due to a preference for physical structure rather than a selectivity for the ethanolamine head group. This hypothesis is supported by additional data in Table I demonstrating that when the substrates were present in 1:1 molar mixtures, differences in the hydrolysis of these two substrates were greatly diminished.

FIG. 5

Effect of pH on secreted PLA_2 activity. Platelet supernatant prepared as described in Fig. 4 was assayed in duplicate for phospholipase A_2 activity at the indicated pH using 1-stearoyl-2-[^{3}H]arachidonyl-GPC (202,000 dpm/trial) as substrate. A buffer containing 100 mM glycylglycine, 100 mM piperazine, and 100 mM sodium formate was substituted for the normal Tris assay buffer. The reactions were stopped by total lipid extraction (13) and the levels of radiolabeled fatty acid were measured following separation by TLC. The data, which are presented as the assay mean ± standard deviation, are representative of 2 separate experiments.

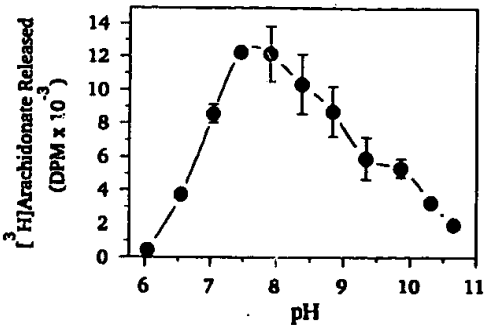


TABLE I

Comparison of PC and PE Hydrolysis by Secreted Phospholipase A_2

Substrate	Arachidonate Released (pmol)			
	PC*	PE*	PC*/PE	PE*/PC
Experiment 1	0.42 ± 0.11	1.92 ± 0.08	0.71 ± 0.15	0.81 ± 0.06
Experiment 2	0.39 ± 0.12	4.82 ± 0.18	0.92 ± 0.10	1.22 ± 0.10

Human platelets (9×10^8 cells/ml) from the Red Cross were incubated in HEPES buffered medium containing 0.5 units/ml of thrombin and 1 mM EDTA for 5 min at 37°C. The phospholipase A_2 activity in the supernatant was then assayed in the presence of 1-stearoyl-2-[^{3}H]arachidonyl-GPC, 1-stearoyl-2-[^{3}H]arachidonyl-GPE, or a 1:1 molar mixture of these two glycerophospholipids in which either the PC or PE was radiolabeled. The concentration of total phospholipid for each trial was 200 pmol/300 µl. Product mass was calculated from the specific radioactivity of the substrate mixtures. The data are presented as the mean from triplicate determinations ± the standard deviation; * denotes the glycerophospholipid labeled with [^{3}H]arachidonate.

DISCUSSION

A number of reports have been published recently demonstrating the secretion of phospholipase A_2 from various cell types. The cell sources and their stimuli have included rabbit peritoneal neutrophils treated with the chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine (21); human neutrophils challenged with ionophore A23187 (22); and sheep (8), rat (9), and rabbit (10) platelets incubated with agents including thrombin, platelet activating factor, ionophore A23187, and ADP. PLA_2 activity has also been observed in the pulmonary secretions of patients with alveolar proteinosis (23); and in the peritoneal exudates of rats (24) and rabbits (25) following intraperitoneal adminstration of caseinate or glycogen, respectively. In the case of rat peritoneal PLA_2, the neutrophil was implicated as the source of the enzyme since a correlation was observed between the number of neutrophils and the PLA_2 activity found in the ascitic fluid (24). Although the secretion of phospholipase A_2 from human platelets was not observed in a previous study (10), the data presented here demonstrate that these cells also release PLA_2 in response to thrombin, collagen, or TPA. In addition to this study, another paper has recently reported that thrombin stimulates PLA_2 release from human platelets (26); however, the secreted PLA_2 in this study was detectable only when radiolabeled *Escherichia coli* membranes were used as substrate, and characterizations of the secreted PLA_2 or the optimal conditions for its release were not reported. To our knowledge, the present study is also the first to document PLA_2 secretion in response to phorbol ester treatment.

Several recent studies suggest that PLA_2 secreted from platelets is identical to intracellular, membrane-associated phospholipase A_2. For example, in both rat (27) and rabbit (10) platelets the secreted and membrane-bound forms of the enzyme exhibit

identical biochemical characteristics. Moreover, comparisons of *N*-terminal or total sequence data for the rat (27,28) and rabbit (10) enzymes have shown complete homology between the secreted and membrane-bound forms. In human platelets, the data to support the existence of a single PLA_2 are less clear-cut. Based on the effects of substrate and Ca^{2+} concentration, the secreted PLA_2 in the present study is very similar to phospholipase A_2 partially purified from detergent-solubilized human platelets (19). However, PLA_2 purified to homogeneity from acid-extracted human platelets (26) requires a 10-fold higher concentration of Ca^{2+} for maximal activation than the secreted PLA_2 (10 mM vs 1 mM). In addition, the secreted PLA_2 has a pH optimum 1-2 units lower than the whole-cell enzyme (16,19,26) and is not stimulated by 1,2-dioleoylglycerol (data not shown), which reportedly enhances human platelet PLA_2 activity by 2-3 fold (19,29).

An interesting observation in the present study is that BSA augments the secretion of PLA_2 from human platelets by nearly 2.5-fold. This effect is saturable at BSA concentrations $\geq$ 0.5 mg/ml. Kramer et al. (16,19) have previously shown that BSA enhances phospholipase A_2 activity isolated from human platelets, presumably by binding to a fatty acid inhibitor (30). As noted earlier, though, the data in Figure 3 represent only the effect of BSA on PLA_2 secretion since all points were assayed at the same BSA concentration. One explanation for this phenomenon may be that BSA prevents binding of the secreted PLA_2 to the sides of the reaction tubes, thereby enhancing its recovery. However, it has also been demonstrated that BSA can extract free fatty acid from biological membranes (31,32). Therefore, an alternative explanation may be that BSA is removing fatty acid or an eicosanoid product from the platelet membrane which otherwise inhibits the secretory mechanism.

Based on the time course of PLA_2 secretion relative to the release of various platelet granule markers, the α-granule has been implicated as the source of the secreted enzyme in rat (9) and rabbit platelets (10). The present findings that PLA_2 secretion from human platelets exhibits a plateau in response to both stimulus dose and time, and that relatively low levels of activity are released, are consistent with a discrete, granule pool of enzyme. In contrast, two earlier papers have reported that stimulated platelets release a large proportion (50-66%) of their total cellular PLA_2 activity (26,27), findings which are not consistent with a specific platelet granule as the sole source of secreted enzyme. However, an inherent complication in comparing secreted to cell-associated phospholipase activities is that platelet pellets contain 80-fold higher levels of phospholipid relative to the supernatants (data not shown). These large differences in phospholipid content could distort a direct comparison of secreted vs. cell-associated PLA_2 activity by altering the specific radioactivity of the substrate, a potential pitfall which may have been overlooked in these earlier studies.

Mizushima et al. (10) have previously demonstrated that the infusion of platelet activating factor into rabbits results in a rapid increase of PLA_2 activity in the plasma. This increase in enzyme activity is paralleled by a significant loss of platelets from the circulation. Therefore, the secretion of PLA_2 by platelets may have *in vivo* significance in the regulation of thrombotic events. As a positive effector, secreted PLA_2 could target other platelets, leading to the hydrolysis of arachidonic acid in the platelet membrane and the generation of TXA_2. As a negative mediator, the PLA_2 could act at the endothelial cell membrane to increase PGI_2 production, thereby serving to limit the area of platelet

deposition during thrombus formation. Due to the prevalence of phospholipase A_2 secretion from platelets of various animal species, these mechanisms warrant further consideration in future studies.

Acknowledgements

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